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Abstract

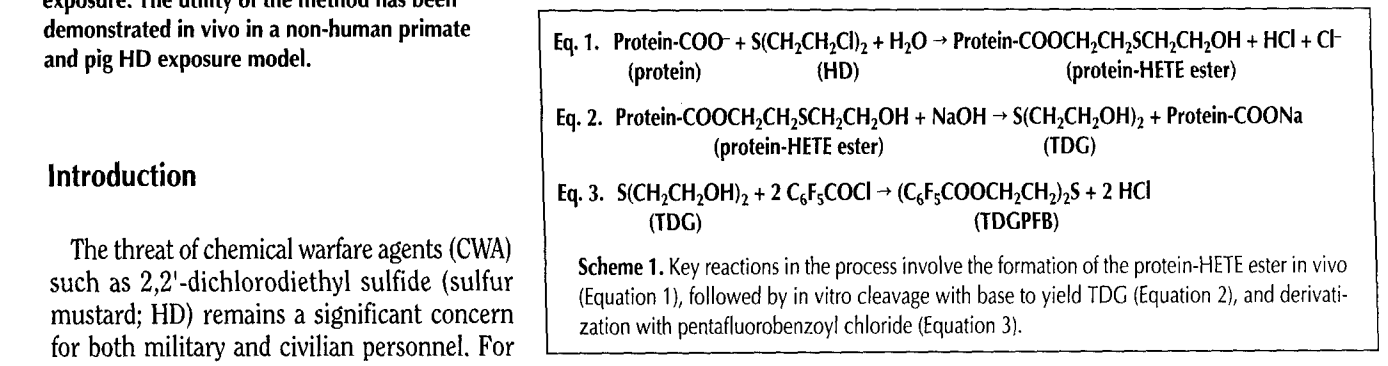
A gas chromatography–mass spectrometry method for determining exposure to the chemical warfare agent 2,2'-dichlorodiethyl sulfide (sulfur mustard; HD) has been developed. The technique is based upon quantitating thiodiglycol (TDG) released from blood protein adducts that are formed upon exposure to HD. Protein was precipitated from plasma, whole blood, or packed red blood cells (RBCs) and then treated with sodium hydroxide to liberate protein-bound TDG. The TDG was derivatized with pentafluorobenzoyl chloride that enabled sensitive detection by negative-ion chemical ionization. Octadeuterothiodiglycol was used as an internal standard. Exposure of human plasma to HD (25nM to 400nM) resulted in a linear relationship (r^2 = 0.9995) between HD concentration and released TDG levels with means ranging from 2.0 to 38 pg/mg protein. The coefficients of variation expressed as a percentage for the data points ranged from 2 to 11.5%. The application of this procedure was demonstrated in two HD animal exposure models. African green monkeys (*Chlorocebus aethiops*) were exposed intravenously to 1 mg/kg HD, and TDG levels in blood samples were analyzed out to 45 days post-exposure. Mean TDG levels were determined to be 220 pg/mg protein on day 1 and declined to 10 pg/mg protein on day 45. Yorkshire cross pigs (*Sus scrofa*) were cutaneously exposed to neat liquid HD, and TDG levels in plasma were determined out to 21 days following exposure. Mean TDG levels were found to be 60 pg/mg protein on day one and decreased to an average of 4 pg/mg protein on day 21. The data from this study indicate that the assay is sensitive and provide a relatively simple approach to assay TDG cleaved from blood proteins at relatively long time frames (21–45 days) after HD exposure. The utility of the method has been demonstrated in vivo in a non-human primate and pig HD exposure model.

Introduction

The threat of chemical warfare agents (CWA) such as 2,2'-dichlorodiethyl sulfide (sulfur mustard; HD) remains a significant concern for both military and civilian personnel. For military personnel, exposures may be intentional as the result of delivery on a battlefield or accidental during demilitarization of chemical weapons stockpiles. Recently, focus on heightened terrorist activity has indicated the risk may expand to include civilian population targets as well. These developments have established an increasing need to develop and improve analytical methodologies that confirm exposure or non-exposure in concerned individuals. For HD, analysis of biomedical matrices for intact agent is not realistic because it interacts rapidly with various tissue components and is present for a relatively short time. Ideally, analytical techniques should provide retrospective detection of HD exposures in that they afford an adequate window of opportunity (i.e., weeks) for sample collection following the suspected event.

Several papers demonstrating assays that enhance analytical methodology to monitor exposure levels of the chemical warfare agent HD in biological matrices have been reviewed (1). These include the immunoassay of HD bound to DNA (2), the gas chromatography–mass spectrometry (GC–MS) assay for the N-terminal valine adduct of hemoglobin (3–5), a GC–MS assay of thiodiglycol (TDG) from skin keratin (5), the liquid chromatography–MS–MS assay of the cysteine adduct in albumin (6), and an immunological method for the detection of mustard adducts to skin tissue (7).

The studies in this current report describe an alternative method for detecting HD exposure. The technique involves the formation and monitoring of TDG cleaved from blood proteins such as albumin and globin (primarily derived from hemo-



Scheme 1. Key reactions in the process involve the formation of the protein-HETE ester in vivo (Equation 1), followed by in vitro cleavage with base to yield TDG (Equation 2), and derivatization with pentafluorobenzoyl chloride (Equation 3).

globin) rather than skin protein (5). Similar to skin keratin, both albumin and globin contain numerous free carboxylic acid groups from aspartic acid and glutamic acid that can be alkylated by the electrophilic HD to give hydroxyethylthioethyl (HETE) esters (Equation 1) (Scheme 1). Theoretically, these reactions would provide a relatively high quantity of blood protein-HD adducts when compared with adducts formed at the four terminal valine sites in globin or the single cysteine site in albumin (1). However, the ester linkages with blood proteins would be vulnerable to catalytic hydrolysis in vivo and potentially lead to a rapid decrease in the concentration of the protein-HETE ester, offsetting any advantage gained by reaction at multiple sites in the proteins. The studies addressed in this report provide some comparisons regarding the retrospective characteristics of the TDG-blood protein method relative to the terminal valine globin and cysteine albumin methods.

The analytical method is based on the key reactions that are shown in Scheme 1. Formation of the protein-HETE ester occurs in vivo (Equation 1). These esters can be readily cleaved or hydrolyzed with dilute base to yield TDG (Equation 2). The TDG can be derivatized with pentafluorobenzoyl chloride to give the bis(pentafluorobenzoyl) ester of thiodiglycol (2,2'-thiobisethanol dipentafluorobenzoate, TDGPFB) (Equation 3) and analyzed by a GC–MS negative-ion chemical ionization (NICI) in the selected ion monitoring mode (5,8,9).

The purpose of this study was to provide preliminary evidence demonstrating the feasibility and simplicity of utilizing the protein-HETE adducts from blood components as a retrospective analysis of HD exposure. Studies were conducted in animal exposure models to demonstrate the utility of the technique and provide insight into the window of opportunity for retrospective confirmation of HD exposure. Herein, we report the details of the analytical procedure, its sensitivity, reproducibility, and application in two HD animal exposure models that characterize the longevity of the protein-HETE esters in vivo.

Experimental

Materials

Ethyl acetate, sodium bicarbonate, anhydrous sodium sulfate, pyridine, and pentafluorobenzoyl chloride were obtained from Sigma-Aldrich (St. Louis, MO) and used without purification. Sulfur mustard was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD); purity as determined by nuclear magnetic resonance spectroscopy was 97.5%. The internal standard octadeuterothiodiglycol (TDG-d_8) was obtained from Ash Stevens (Detroit, MI). Analysis of TDG-d_8 by GC–MS demonstrated no evidence of undeuterated material that could potentially interfere with assay of the native compound. Bond Elut silica 100-mg Si SPE cartridges (Varian, Harbor City, CA) were conditioned with 1 mL of ethyl acetate before use. Pooled human plasma with sodium citrate added was obtained from Innovative Research (Southfield, MI). Analysis of this plasma with the procedure described herein demonstrated no evidence of the protein-HETE adducts.

Animals

Pig and monkey blood were obtained from experimental animals used at the U.S. Army Medical Research Institute of Chemical Defense in Institute Animal Care and Use Committee (IACUC) approved protocols. Animals were maintained and used under a program accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC International).

In total, six African green monkeys (*Chlorocebus aethiops*) were utilized in these studies. The animals were anesthetized with ketamine and weighed, and a catheter was introduced into the saphenous vein for HD administration. The appropriate HD dose (1 mg/kg) was prepared in saline and administered as two half-dose injections infused over 2.5 min with a microprocessor controlled infusion pump (model 200P, Stoelting Co., Wood Dale, IL). Each HD injection was followed with a 1.0 mL saline flush. At the appropriate times after exposure, the animals were anesthetized with ketamine, and blood samples were obtained from the saphenous vein. Sample volumes used in these studies were 0.25 mL for whole blood and 0.5 mL for packed red blood cells (RBCs).

Two specific pathogen-free Yorkshire cross weanling pigs (*Sus scrofa*, 9–11 kg) were utilized in this study. Six exposure sites were set up on the ventral abdominal surface of each animal, three sites per side parallel to and approximately 2.5 cm lateral to the teat line and located between the axillary and inguinal area. For HD exposure, 400 µL of undiluted (neat liquid) HD was applied to 3-cm diameter dosing templates placed in the center of each exposure site. The three sites on the left side of pig #1 were exposed to HD for 30 min, and the three sites on the right side exposed for 8 min. Exposure times were reversed for pig #2. Details of the exposure technique have been previously described (10,11). Blood samples were collected from the anterior vena cava while the animals were under a surgical plane of anesthesia before agent exposure (day 0) and on post-exposure days 1, 2, 7, 14, 21, and 28. Blood samples (2 mL) were placed into tubes coated with an ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Plasma was separated from whole blood by centrifugation for 20 min (2000 × *g*) at 5°C.

Sample preparation

Sample volumes used for the NHP studies were 0.25 mL (whole blood) and 0.5 mL (packed RBCs); samples from pig studies were 1.0 mL (plasma). Plasma protein was precipitated, washed with acetone, and then washed with diethyl ether according to previously published methods (3). For whole blood or RBC samples, the procedure was identical except that 1% HCl in acetone was used for the precipitation step to remove heme from hemoglobin (3). After drying at room temperature, the precipitated protein was weighed to approximately 20 mg (all TDG concentrations were expressed as mass TDG/mg protein) and then treated with 1M NaOH (200 µL) and heated at 70°C for 90 min. After neutralization using 3N HCl (60–70 µL), the sample was dried with sodium sulfate (200 mg), followed by extraction with either propyl acetate or ethyl acetate (1.1 mL). A portion of the organic layer was removed (500 µL); to it was added the internal standard TDG-d_8 (10 µL; 1 µg/mL), and sodium sulfate (200 mg) to promote further drying. To

form TDGPFB and TDG-d_8PFB, pyridine (15 µL) and the derivatizing compound pentafluorobenzoyl chloride (20 µL) were added to the organic phase, and the solution was shaken for 10 min. Water (20 µL) and sodium bicarbonate (20 mg) were added to the mixture to neutralize any residual derivatizing agent. The ethyl acetate layer was isolated and dried over sodium sulfate (200 mg); then a portion (200 µL) was filtered over a pre-conditioned SPE Si cartridge. An additional 400 µL of ethyl acetate was passed through the SPE cartridge, and the combined fractions were analyzed using GC–MS.

Initial studies were conducted to determine the effect of incubation time on TDG recovery from spiked plasma samples. For these experiments, HD was spiked into pooled human plasma to produce a final concentration of 4µM (5 mL). The spiked human plasma was divided into two equal-volume (2.5 mL) samples. They were both incubated at 37°C with gentle shaking in a water bath, one for 5 h and the other for 25 h. Subsequent to incubation, protein from the samples was obtained, treated, and analyzed as described except that the internal standard was added prior to the extraction step.

Standard curves were prepared to establish the relationship between HD concentration in spiked human plasma and amount of TDG assayed following cleavage from proteins. For these studies, a standard curve (consisting of five concentration levels of HD) was prepared and assayed on six different days. The standard curves were prepared as follows. Sulfur mustard (100 µL; 40µM) in saline was added to 9.9 mL of human plasma. This spiked plasma sample was then serially diluted with human plasma; the final HD concentrations studied were 400, 200, 100, 50, and 25nM. One milliliter of plasma was used at each concentration to obtain protein. The samples were incubated at 37°C with gentle shaking for 18 h. Protein from the plasma standards was obtained, treated, and analyzed as described.

Instrumentation

GC–MS separations were performed on an Agilent 6890 GC interfaced to an Agilent 5973 mass-selective detector. The GC was fitted with a DB-5MS bonded phase capillary column (30 m × 0.25-mm i.d., 0.25-µm film thickness, J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a column head pressure of 12 psi. The oven temperature was held initially at 80°C for 1 min, programmed from 80 to 225°C at 30°C/min, and held at 225°C for 11 min. Splitless injections of 1-µL volume were made using an Agilent 7683 autosampler. The injection port temperature was set at 250°C, split vent delay at 1 min, and the transfer line temperature at 280°C. Typical retention times were 13.3 min for TDG-d_8PFB and 13.5 min for TDGPFB.

The MS analysis was conducted using NICI with methane as the reagent gas. The resolution capability of the Agilent 5973 mass selective detector is specified as unit mass. The source and quadrupole temperatures were set at 150 and 106°C, respectively. The molecular ion and the M+1 isotope ion were monitored for the TDGPFB (*m/z* 510 and 511) and TDG-d_8PFB internal standard (*m/z* 518 and 519). Relative to monitoring a single ion, two ions were used to enhance sensitivity and to provide further confirmation of peak identity. The dwell time for each ion was 100 ms, resulting in a total scan rate of 2.15 cycles/s. The electron multiplier voltage was set at +400 volts relative to the autotune setting. Analysis of the purified TDGPFB indicated that the lower limit of detection was approximately 10 pg/mL (signal/noise = 3:1).

Data analysis

Graphic representations, linear regression analyses, and non-linear curve fitting analyses were carried out with GraphPad Prism software (version 3.02, 2000, GraphPad Software, San Diego, CA). Concentration-time data were fit to a one- or two-phase exponential decay equation. Selection of the best-fit equation was accomplished by comparison with an F test. Statistical significance was considered at a level of $p < 0.05$. Calculations for TDG concentrations and other descriptive statistics were accomplished with Microsoft Excel 2000. Ions used for quantitation purposes were *m/z* 518 and 519 for the internal standard and *m/z* 510 and 511 for the analyte.

Results and Discussion

The focus of this study was to demonstrate the feasibility and simplicity of the assay for TDG hydrolyzed from protein-HETE esters in blood following an HD exposure. Additionally, studies were conducted in animal exposure models to demonstrate the applicability of the technique to measure TDG cleaved from protein-HETE esters derived from whole blood, plasma, and RBCs. These studies also provide insight regarding the window of opportunity for this method for the retrospective detection of HD exposures.

Studies examining the effect of incubation time on TDG recovery from spiked human plasma indicated that both incuba-

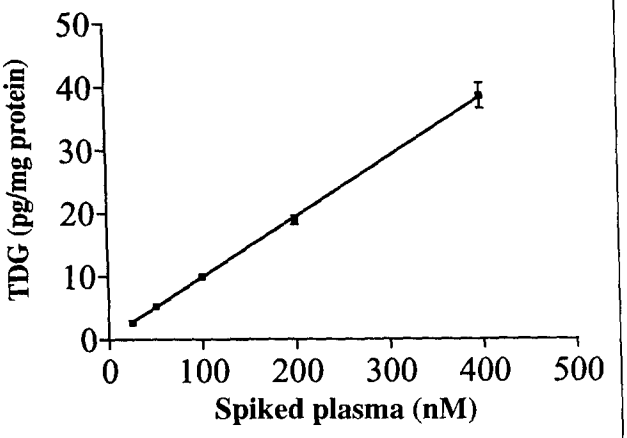


Figure 1. The relationship between TDG assayed (i.e., cleaved from protein-HETE esters) as a function of HD concentration in spiked human plasma is presented. The quantity of TDG was determined from the analysis of human plasma spiked with concentrations of HD that varied from 25nM to 400nM (r^2 = 0.9997). Each data point represents the mean of six separate assays ± standard deviation; CVs ranged from 2 to 11.5%.

tion times examined (5 or 25 h) yielded similar results. The analyses resulted in values of 585 and 587 pg TDG/mg protein for the 5- and 25-h incubation times, respectively. These data indicate that the hydrolysis of the protein-HETE esters in spiked plasma did not appear to play a critical role because the values for TDG were similar for both incubation times examined. Based on these data, all subsequent sample analyses were performed using 18 h as the incubation time.

Data indicating the relationship between mean TDG assayed (i.e., cleaved from protein-HETE esters) as a function of HD spiked human plasma over a 16-fold range (25 to 400nM) are summarized in Figure 1. The correlation coefficient (r^2) of the mean TDG value assayed over six days as a function of spiked plasma concentration was 0.9997. The r^2 value obtained when plotted as individual data points as a function of spiked plasma concentration was 0.9951. This is consistent with previously reported values of other protein-HD adducts (3–6). The percent coefficients of variation (%CVs) for the mean TDG concentrations (n = 6) at various spiking concentrations ranged from 2 to 11.5%. The lower limit of TDG detection of 2 pg/mg protein at the 25nM exposure level indicated a sensitivity four times greater than the limit of detection of the valine adduct assay at 100nM SM in vitro exposure of blood (3).

The assay was also evaluated in two in vivo studies. The first involved the intravenous (iv) administration of HD (1 mg/kg) to five African green monkeys, and the second used a cutaneous neat liquid HD exposure to two weanling pigs. The results from the NHP study (n = 5) depicting the mean levels of TDG cleaved from whole blood proteins as a function of time are shown in Figure 2 (solid squares). Also shown in Figure 2 is the computer-generated two-phase exponential decay curve (solid line) that was fit to the concentration-time data. The data indicate that measurable quantities of TDG could be released from proteins for at least 45 days after the exposure. The rate of loss of the protein-HETE ester with time was greater than the turnover rate for the blood proteins. Albumin has a half-life ($t_{½}$) of 20 to 25 days, whereas the $t_{½}$ for the decrease in the concentration of protein-HETE ester was calculated to be 14.95 days for the terminal decay phase. This shorter $t_{½}$ was consistent with appreciable protein-HETE hydrolysis in addition to normal albumin turnover. Thiodiglycol, either free or from in vivo hydrolysis of the ester, would not be measured in this assay because the precipitated protein is washed thoroughly prior to analysis. However, even with hydrolytic loss of the bound TDG, the high sensitivity of the method enabled the detection of TDG for up to three $t_{½}$s or 45 days. Although this is shorter than the 90-day period observed with the valine assay, differences may be accounted for by species variation (i.e., marmoset) as well as by the larger dose of HD (4 mg/kg, iv) utilized (1).

The curve depicting the decay of the mean values of TDG as a function of time from the NHP study demonstrates relatively

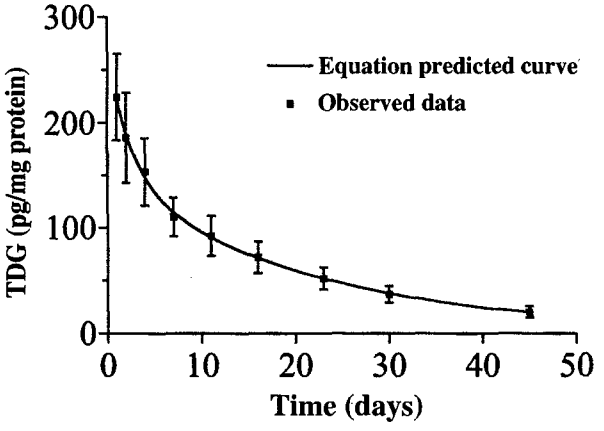


Figure 2. The mean levels of TDG cleaved from whole blood proteins as a function of time are presented from the monkey study. The data depicting the mean TDG levels obtained from the blood protein following a single intravenous exposure to HD (1 mg/kg) are shown (solid squares). The data are the mean (n = 5) values ± standard deviation. Also shown is the computer-generated two-phase exponential decay curve (solid line) that was fit to the concentration-time data.

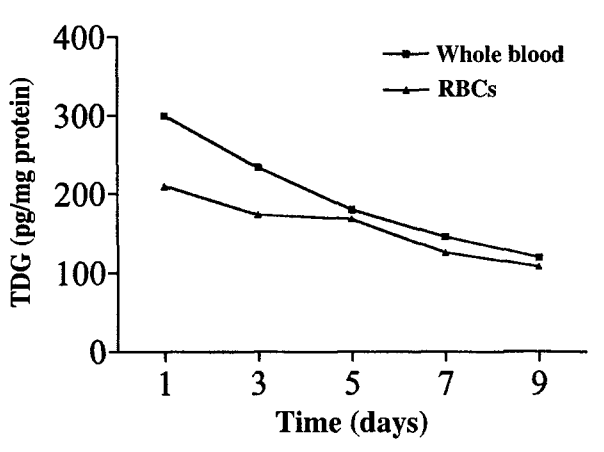


Figure 3. The relationship between quantities of TDG obtained from analysis of whole blood or packed RBCs in an NHP is shown. Thiodiglycol was cleaved and analyzed from whole blood or RBC protein from one animal according to the described methodology following an iv dose of HD (1 mg/kg).

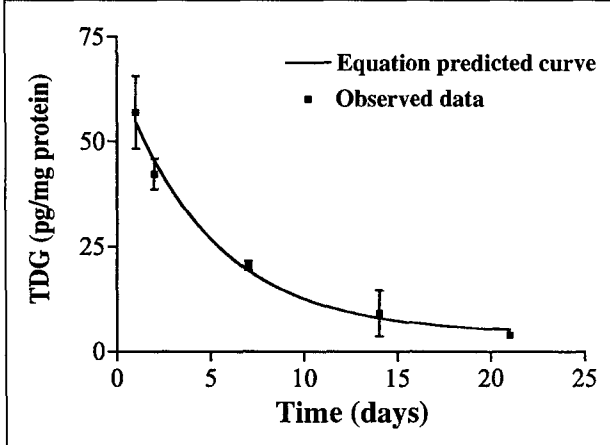


Figure 4. The concentration-time course of TDG cleaved from plasma proteins in weanling pigs is presented. Mean TDG levels obtained from the plasma of two pigs following a cutaneous exposure of neat liquid HD (2.4 mL/pig) over a 21-day period are shown (solid square). The data are the mean values from two animals run in duplicate ± standard deviation. Also shown is the computer generated one-phase exponential decay curve (solid line) that was fit to the concentration-time data

small variation as reflected by the %CVs. Across the time points, the %CVs ranged from 16.7 to 26.3%. The observed data reflect both interanimal variability and errors inherent to the analytical procedure (2 to 11.5%). As a consequence, it is reasonable to note that the level of HD binding to protein carboxylate sources in the blood of the five animals is more consistent than indicated by the errors depicted in Figure 2.

Further studies were carried out in a sixth monkey to demonstrate the utility of this methodology using both packed RBCs and whole blood (Figure 3). The data indicate that the values of TDG from RBCs over the first nine days of the study were lower than the TDG values obtained from the whole blood of the same animal, particularly during the early time period of three days after exposure. Therefore, TDG values per mg of plasma protein are probably greater than TDG values per mg of globin from the RBC. However, the difference is relatively small, indicating that plasma, whole blood, or RBCs can be used in the assay and yield similar results.

The second in vivo application of the assay focused on the quantity of TDG recovered from plasma protein after a single 2.4-mL (0.4 mL used at each of six sites) cutaneous neat liquid HD exposure to two pigs. The TDG level was determined at six different time points over a 28-day period. The levels of TDG cleaved from plasma proteins as a function of time are presented along with the computer-generated predicted curve for a one-phase exponential decay in Figure 4. Based on the exponential decay curve, the $t_{½}$ was calculated to be 3.9 days. The initial day one average value of TDG (60 pg/mg protein) decreased to an average of 4 pg/mg protein at day 21. Day 28 values were essentially the same as the background, so only data to day 21 following exposure are presented. The %CVs were similar to those obtained in the NHP study and ranged from 5.2 to 18.2%.

Conclusions

An assay for the detection of HD exposure based on the analysis of TDG liberated from blood proteins has been reported. The versatility of the technique has been demonstrated by its ability to analyze TDG cleaved from proteins derived from whole blood, plasma, or globin from RBCs. Additionally, this method was applied to the analysis of HD exposure in two different animal models. These analyses detected released TDG in whole blood from African green monkeys for up to 45 days after iv HD exposure and 21 days after cutaneous liquid exposure to weanling pigs. These data indicate that the method is relatively simple, sensitive, and reproducible and will potentially provide a relatively long window of opportunity for the retrospective detection of HD exposure.

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